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Research article

Fungal treatment of agricultural washing wastewater: Comparison between two operational strategies

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ABSTRACT

Agricultural washing wastewater (AWW) is an important source of pesticides that, given its intrinsic characteristics, has a high potential to be treated by fungal bioremediation using white rot fungi. In the present study, two AWW treatment strategies were compared: a fluidized-bed reactor (FBR) with *T. versicolor* pellets and a rotating drum bioreactor (RDB) with *T. versicolor* immobilized on wood. The RDB effluent showed better results in all studied parameters compared to those of the FBR, including pesticide removal (87%), toxicity, laccase activity, COD, absorbance and microbial communities. Additionally, the fungal assemblage showed that *T. versicolor* was successfully immobilized in the RDB, which triggered a major shift in the initial community. Afterwards, solid by-products were treated in a fungal biopile-like system reaching high biodegradation rates. Therefore, this study validates the fungal RDB as a viable alternative for AWW treatment, opening up the possibility of a further *in-situ* and full-scale application.

1. Introduction

The main effects of climate change, i.e., growth in atmospheric CO_2 levels, temperature rise and changes in precipitation patterns, have been shown to lead to increased pest activities on crops. Changes in the dynamics of pest influx and crop disease threaten to cause significant agricultural production and economic losses, and compromise global food supply and security (Singh, 2015). In addition, a growing world population and rising living standards require a greater effort from the primary sector to meet global food demand. In this context, the use of pesticides is necessarily unavoidable to ensure pest control and food production worldwide (Popp et al., 2013).

Nevertheless, pesticides are becoming a major global concern owing to their overuse in agricultural activities, environmental persistence, high mobility, bioaccumulation and toxic effects (Sharma et al., 2019). For these reasons, good practices in the agricultural sector should be subjected to clear regulations regarding the correct management and application of pesticides, comprising the prohibition of highly toxic pesticides, the selection of specific pesticides for each type of pest and crop, and the application of reasonable doses. In this respect, particular emphasis should be placed on agricultural washing wastewater (AWW), also known as rinsate, which is generated on farms when washing agricultural machinery and equipment. Afterwards, AWW is usually deposited in collection ponds, avoiding dilution by rainfall and leaks during handling that could contaminate the surrounding soil (European Commission, 2009a). Finally, accumulated AWW should be treated by an *in-situ* process specifically designed for this application before discharge.

Over the last few decades, various physical, chemical and biological technologies have already been implemented for pesticide elimination from wastewater. However, there are several limitations that affect the treatment of pesticides by physical-chemical methods, such as the transfer of these compounds from liquid to solid phase without real degradation (e.g. sorption), high operational costs (e.g. membrane filtration) and the potential formation of transformation products (e.g. ozonation) (Ahmed et al., 2017). Among them, sorption has been one of the most widely applied techniques for pesticide removal due to its easy manipulation, high removal efficiency and relative low cost (depending on the sorbent), whereas this technology has significant drawbacks such as critical pH dependence and difficulties in sorbent preparation and regeneration (Mojiri et al., 2020; Titchou et al., 2021).

In contrast, bioremediation is gaining increasing attention in recent

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decades as an environmentally friendly, efficient and low-cost approach (Marican and Durán-Lara, 2018). In particular, bioremediation using white rot fungi (WRF) seems to be a very promising technology for AWW treatment. AWW is usually produced at relatively low volumes, which is desirable in biological processes using WRF as these microorganisms generally require relatively long hydraulic residence times. Furthermore, AWW is generated at high concentrations of multiple pesticides, which can be treated by WRF due to their excellent resistance to elevated toxicities. Consequently, WRF do not require prior acclimatisation as in other biological processes such as conventional activated sludge. The powerful enzyme system of WRF allows the removal of a wide range of organic compounds, including pesticides, considered recalcitrant in other treatment systems (Mir-Tutusaus et al., 2018). Among WRF, *Trametes versicolor* has recently proved to be one of the best candidates in terms of degradation activity for several pesticides (Hu et al., 2020).

Fungal reactors have been extensively studied for wastewater treatment (Espinosa-Ortiz et al., 2016). During the last years, the scientific community seems to have reached a certain consensus on the benefits provided by biomass immobilization in fungal reactors, as it overcomes some of the most common limitations in fungal treatments, including fungal growth on reactor walls and accessories, foam production and biomass washout by decoupling the cellular retention time (CRT) from the hydraulic retention time (HRT) (Mir-Tutusaus et al., 2018). There are basically two types of fungal immobilization: autoimmobilization as pellets and immobilization on carriers. Carriers can be either inert (e.g., polyurethane foam cubes) or non-inert (e.g., wood) (Zhuo and Fan, 2021).

In this regard, one potentially applicable system for AWW treatment is an air-pulsed fluidized-bed reactor (FBR) using *T. versicolor* pellets, which is a well-established fungal reactor that has been successful in treating micropollutants at lab-scale even for a long-term period (Mir-Tutusaus et al., 2019). However, operating an FBR under non-sterile conditions has several operational limitations, such as bacterial contamination (since glucose is added as a carbon source) and foaming (as forced aeration is required) (Mir-Tutusaus et al., 2018).

Alternatively, a rotating-drum reactor (RDB) with *T. versicolor* immobilized on wood has shown good performance in treating pesticides from agricultural wastewater and its long-term viability has also been previously demonstrated (Beltrán-Flores et al., 2020). Immobilization on lignocellulosic materials such as wood has some advantages: sustainability, low cost, use of a specific substrate for fungi (limiting bacterial competition) and micropollutant sorption (Hu et al., 2020; Torán et al., 2017). Furthermore, the design of the RDB has been specifically conceived for *in-situ* application in agricultural fields. However, special emphasis should be placed on the DOC increase resulting from the release of soluble organic compounds into the water and the sorption capacity of wood (Beltrán-Flores et al., 2022). The RDB is a bioremediation system where very complex physicochemical and biological interactions coexist, thus pesticide removal can occur by different mechanisms: sorption on wood, sorption by the fungal biomass and biodegradation by the fungal enzyme system (both extracellular and intracellular). In this regard, previous studies have evaluated the removal kinetics and mechanisms of various pesticides by wood and *T. versicolor* (Beltrán-Flores et al., 2021; Hu et al., 2022).

Therefore, a comparative study between both reactors is required to elucidate whether fungal immobilization strategy, i.e., pellets (autoimmobilization) or immobilization on lignocellulosic material, is more viable for AWW treatment. The aim of the present work was to set up an FBR and an RDB using *T. versicolor* as inoculum for treating AWW from pesticide application equipment, and compare their performances and applicability considering different perspectives.

2. Materials and methods

2.1. Fungal strain and reagents

T. versicolor ATCC 42530 was acquired from the American Type Culture Collection. The fungus was maintained on malt extract (2% w/v) at 25 °C and routinely subcultured every 30 days. Blended mycelial suspension and *T. versicolor* pellets were prepared as previously described elsewhere (Blánquez et al., 2004). *T. versicolor* immobilized on *Q. ilex* wood chips was prepared as reported by Beltrán-Flores et al. (2021). Acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). These chemicals and others used for *T. versicolor* subculture were of high purity grade.

2.2. AWW characterization

AWW was taken in September 2020 from an artificial pond designed to collect wastewater produced after washing spraying equipment and agricultural machinery in the Sustainable Plant Protection program of the Agrifood Research and Technology Center in the Institute of Agrifood Research and Technology (IRTA) in association with the Mas Badía Foundation (Catalonia, Spain). Wastewater was stored in the fridge at 4 °C until use.

2.3. Bioreactor setup

2.3.1. Rotating drum bioreactor

The graphical abstract shows the RDB, whose characteristics have been described in a recent publication (Beltrán-Flores et al., 2022). The RDB (2.3 L) was fed in sequential batches for two periods of 17 days and the inner tube was rotated one and a half turns every 24 h. An external recirculation loop (4.7 L day^{-1}) was required for pH adjustment and DO measurement, which were performed in a recirculation tank (≈0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. The DO level was monitored using a CyberScan 600 Series Waterproof Handheld (Eutech Instruments). The DO level remained above 30% in the recirculation tank throughout the treatment. The inner tube contained 1100 g DW of colonized wood. Liquid samples were taken from the reactor effluent for analyses of pesticide concentration, laccase, absorbance, chemical oxygen demand (COD), heterotrophic plate count (HPC), toxicity, phytotoxicity and microbial communities. Solid samples were withdrawn at the end of the treatment to measure the remaining pesticide content and microbial communities. The removal efficiency of the studied pesticides was calculated according to Eq. (1):

$$Pesticide\ removal\ (\%) = \frac{C_0 - C_t}{C_i} \cdot 100 \quad (1)$$

Variables: C_0 is the initial concentration (or content inside the wood) of the studied pesticide (mg·L^{-1} for the liquid phase and mg·g^{-1} for the solid phase) and C_t is the concentration (or content inside the wood) at a specific time (mg·L^{-1} for the liquid phase and mg·g^{-1} for the solid phase).

2.3.2. Fluidized-bed reactor

A total of 2.2 g L^{-1} DW pellets (WW/DW = 23 ± 3 for triplicate measurement) were transferred to an FBR (see graphical abstract) consisting of a 7.5 cm diameter body and a considerably wider 13.5 cm diameter head. The FBR useful volume was 1.5 L, but only 1.3 L of AWW was added in anticipation of possible foaming. The AWW was previously autoclaved at 121 °C for the experiment under sterile conditions. The reactor was operated in batch mode for 17 days at 25 °C. The pH was controlled at a constant value of 4.5 by adding 1 M NaOH or 1 M HCl. Fluidized conditions were achieved by introducing a 1 s air pulse every 4 s through a solenoid valve placed at the bottom part of the reactor. The aeration rate was set at 0.8 L min^{-1}. Glucose and NH_4Cl were fed for

T. versicolor maintenance at a molar C/N ratio of 7.5 (Mir-Tutusaus et al., 2017). The same analyses performed for the liquid samples in the RDB (see Section 2.3.1) were replicated for the FBR.

2.4. Solid-phase treatment in a biopile-like system

Solid by-products produced in the RDB were piled inside Scott glass bottles (Duran, Inc; 250 mL, 95 × 105 mm) equipped with an open-port screw cap opened with a passive air inlet through a 0.45 μm filter. A total of 30 g DW of by-products (in triplicate) obtained at the end of the second batch in the RDB were treated under non-sterile conditions and 25 °C for 27 days. Afterwards, the solid samples were withdrawn to measure the remaining pesticide content.

2.5. Pesticide identification and quantification

A proposal for the identification of the pesticides detected in the agricultural wastewater was performed by high-performance liquid chromatography (HPLC, 1200RR, Agilent Technologies). The HPLC was equipped with a diode-array detector (DAD) and a microTOF-Q Mass Spectrometer (Bruker Daltonics, Bremen, Germany). Briefly, 1 mL of sample was withdrawn and filtered through a 0.22 μm Millipore Millex-GV PVDF filter. Chromatographic separation was performed using an C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 5 μm, 4.6 mm × 150 mm) with the following operating conditions: 30 °C, eluent flow rate of 0.9 mL·min-1 and injection volume of 40 μL. The mobile phase consisted of 0.01% formic acid solution and acetonitrile, which was pumped isocratically at a ratio of 50:50.

Analytes were first detected by DAD using a wavelength of 252 nm. Afterwards, the eluent was split using a T-type splitter before entering the QTOF (split = 1:2). Thus, the flow rate reaching the ESI-Q-TOF-MS detector was 0.3 mL min^{-1}. Samples were analysed in the positive electrospray mode (ESI). MS data were acquired at a m/z range of 50–1000 Da. Spray voltage was set at 2000 V and the drying gas temperature was 210 °C. The capillary voltage of the ion source was 5000 V. The dry gas flow rate was set at 8.0 L min^{-1} and the nebulizer gas pressure was 4.0 bar. Nitrogen was used as both drying and nebulized gas. The pre-pulse storage time was 8 μs and the source transfer time was 65.0 μs Instrument calibration was performed by using a 10 mM sodium formate solution.

The MS data were processed using Bruker Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany). Candidate compounds were identified on the Chemspider website by their exact masses (Royal Society of Chemistry, 2022). Finally, confirmatory analyses and quantification were conducted using the analytical standards of the detected pesticides. In this case, the analysis was performed using the same analytical method in a Dionex Ultimate 3000 HPLC-UV.

2.6. DNA extraction and PCR-DGGE of the fungal community

All liquid and solid samples were collected, immediately stored at 4 °C, and processed as described earlier by the authors (Beltrán-Flores et al., 2022). Briefly, total DNA extraction was performed on the samples, and the DNA extracts were subsequently amplified by nested PCR to reduce non-specific products and increase sensitivity, using two sets of specific primers targeting fungal ITS (Internal Transcribed Spacer) region (Gardes and Bruns, 1993; White et al., 1990). Afterwards, all amplification products were subsequently studied by denaturing gradient gel electrophoresis (DGGE) to assess fungal genetic variations throughout the experiment and provide an estimate of richness and abundance of prominent members of the fungal assemblage.

After visualization of DGGE profiles, prominent bands were cut, recovered, and sequenced at external facilities by Macrogen, Inc. (South Korea). Sequences determined in this study are available at the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers ON116618 through ON116654. The Vegan package was used to carry out non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance and similarity indices (Oksanen et al., 2020). Colour, Bacterial counts, COD and diversity indexes were further visualized by NMDS using envfit function ($p_{max} = 0.05$).

2.7. Vibrio fischeri bioluminescence inhibition test (Microtox® test)

Toxicity test was performed using the acute toxicity bioassay kit from Modern Water (London, UK). In brief, the test is based on the attenuation of *Vibrio fischeri* bioluminescence after 5 and 15 min of exposure to selected dilutions of the samples, previously adjusted to pH 7. Toxicity was expressed as toxicity units (TU).

2.8. Seed germination test

The phytotoxicity of the treated/untreated agricultural wastewater was analysed through a seed germination test of tomato (*Solanum lycopersicum*) by exposing 10 seeds to 10 mL of each sample (triplicate) in Petri dishes for 24 h. Subsequently, each group of 10 seeds was placed on a Whatman Nº1 filter of 70 mm diameter inside Petri dishes for 10 days of incubation at room temperature and exposure to natural light. The filters were pre-humidified with 3 mL distillate water. Relative seed germination (SG), relative root elongation (RE) and germination index (GI) were determined as described elsewhere (Rodriguez-Rodriguez et al., 2011).

2.9. Other analyses

Pesticides were extracted from the wood as recently reported (Beltrán-Flores et al., 2022). Ergosterol analysis was performed by the homogenization and extraction of the colonized wood per triplicate, as described by Rodríguez-Rodríguez et al. (2010a). Conductivity was measured using a CRISON MicroCM 2100 conductometer. Colour was determined at a wavelength of 650 nm with a UNICAM 8625 UV/VIS. COD and ammonia concentrations were measured using the commercial kits LCK114 or LCK314m and LCH303, respectively (Hach Lange, Germany). Total suspended solids (TSSs) and volatile suspended solids (VSSs) were measured according to the standard methods 2540 D and 2540 E, respectively, and HPC were determined following the standard method 9215 (Rice et al., 2017). Chloride, nitrite and sulfate concentrations were quantified by ion chromatography using a Dionex ICS-2000.

T. versicolor superficial growth on wood was evaluated by scanning electron microscopy (SEM). Solid samples were fixed on a 2.5% glutaraldehyde solution with 0.1 M phosphate buffer (PB). Post-fixation was performed using 1% osmium vapour containing 0.8% ferrocyanide in 0.1 M PB overnight. Afterwards, the samples were washed with ultrapure water and dehydrated in increasing concentrations of ethanol (50, 70, 90, 96 and 100%). Finally, the samples were subjected to the critical point drying method (Baltec CPD030) and metallized with AuPd.

3. Results and discussion

3.1. AWW characteristics

Physicochemical characteristics of the AWW are summarized in Table S1 (Supplementary material). The main characteristics of this AWW compared to other agricultural and urban wastewater was the high content of organic matter in the form of COD or TOC detected, and as a consequence of the eventual decomposition of this organic matter, slightly higher levels of ammonium and bacteria counts (Beltrán-Flores et al., 2021; Metcalf and Eddy, 2003). This study analyses not only the treatment efficiency concerning the removal of pesticides, but also the evolution of other characteristic parameters to determine the final effluent quality.

3.2. Pesticide identification and quantification

Liquid chromatography coupled to time-of-flight mass spectrometry (LC-qTOF-MS) is a useful technique for qualitative analysis and identification of organic micropollutants (Arsand et al., 2018). High mass resolution along with the acquisition of mass profile data allowed the screening of non-targeted compounds.

Unknown compounds were identified through exact mass analysis. LC-qTOF-MS analysis shows high specificity, allowing the pesticide identification by the characterization of the monoisotopic mass with a maximum error of ±2.5 mDa. The detected exact masses were close to those of the following common pesticides: thiacloprid (THIA), chlortoluron (CHLOR), azoxystrobin (AZO) and tebuconazole (TEBU). Moreover, the isotope pattern validated the identification of such pesticides. In this regard, the experimental isotopic patterns match the predicted isotope distributions as shown in Table S2 (Supplementary materials).

Afterwards, commercial reagents of each pesticide were spiked to the AWW to verify that the chromatogram peaks of the standard compounds matched those detected in the original matrix. After confirmatory analysis, initial pesticide concentrations were quantified obtaining a total of 40.55 mg L^{-1} of pesticides, consisting of 19.17 mg L^{-1} THIA, 7.42 mg L^{-1} CHLOR, 4.47 mg L^{-1} AZO and 9.49 mg L^{-1} TEBU. Although these concentrations are relatively high for agricultural water (Köck-Schulmeyer et al., 2019), they are reasonable considering that the AWW comes from the washing of agricultural equipment that had been in direct contact with pesticides. THIA is a neonicotinoid insecticide that acts by disrupting the nervous system of these organisms. This pesticide was included in the watch list of substances in Decision (2015)/495/EU (European Commission, 2015), as it was suspected to pose a high risk to human health as endocrine disrupting, neurotoxic and carcinogenic compounds. Recently, the EC banned THIA by not renewing its license for use in the EU (European Commission, 2019). CHLOR is an herbicide belonging to the phenylurea class that acts by the inhibition of photosynthetic electron transport. It is moderately toxic to most aquatic species, birds and worms, although toxicity to mammals is considered low. AZO is a broad-spectrum systemic fungicide that acts on the target organism by inhibiting spore germination and has been recognized to have physiological impact on some aquatic organisms. TEBU is a triazole fungicide that affects the biosynthesis of the phytohormone gibberellin, thus inhibiting seed germination and plant growth. It is classified as a possible carcinogen (rating C) on the U.S. Environmental Protection Agency's Office of Pesticide Programs list of carcinogens. These last three pesticides are currently approved for use in the EU under EC Directive 1107/2009 (European Commission, 2009b), but AZO and TEBU are currently under study. AZO has been included in the watch list of substances in Decision (2022)/1307/EC and TEBU was first included in the watch list of substances in Decision (2020)/1161/EC, but due to insufficient high-quality monitoring data TEBU has remained on the watch list in Decision (2022)/1307/EC. (European Commission, 2022;2020).

3.3. Comparison between bioreactors

3.3.1. Pesticide removal performance

Two different fungal immobilization strategies were used: auto-immobilization (pellets) in the FBR and immobilization on wood chips in the RDB. In the RDB, the initial ergosterol content was approximately 0.121 ± 0.018 mg g DW^{-1} of wood. Considering that 1100 g of colonized wood chips were transferred to the reactor and the ergosterol-biomass correlation for the selected strain is 6.61 mg g DW^{-1} of fungal biomass (Rodríguez-Rodríguez et al., 2010b), a total of 20 g DW of biomass were introduced to the reactor. However, only 30% of the biomass was continuously submerged in the reactor, which is 2.2 g biomass $DW{\cdot}L^{-1}$. Hence, an equivalent pellet inoculum was used in each FBR experiment.

Pesticide removal profiles in the FBR and RDB treatments are shown in Fig. 1. The FBR operation was conducted under both sterile (FBR-S) and non-sterile (FBR) conditions to evaluate the effect of microbial contamination on the treatment performance. Although non-sterility can benefit the efficiency of fungal treatment owing to the positive synergies created by the microbial consortium, e.g. by metabolite degradation, non-sterile conditions can also exert pressure on the WRF survival, which in turn can reduce the fungal activity and process duration (Mir-Tutusaus et al., 2018). In this case, the FBR-S was more efficient than the FBR concerning total pesticide removal, reaching 88 and 51%, respectively. This result confirms that other microorganisms, such as bacteria, can compete for substrate and reduce fungal metabolic activity over time (Mir-Tutusaus et al., 2019). However, from a practical point of view, the FBR can be considered a better option than the FBR-S given that sterilisation is a costly process that should be avoided at full-scale applications, and negligible bacterial proliferation was observed in the FBR (see Section 3.3.3.) while maintaining high removal efficiency.

Alternatively, the AWW was also treated by *T. versicolor* immobilized on wood in an RDB, which has shown good results in treating agricultural wastewater under non-sterile conditions (Beltrán-Flores et al., 2022). As shown in Fig. 1, higher removal yields were obtained in the RDB, reaching up to 85% of total removal, compared to the FBR (51%). In fact, equilibrium was not reached in the FBR during the 17 days of treatment, while the RDB showed maximum removals after the first 5 days. This result is particularly interesting for future full-scale reactor applications, as operating periods, and thus reactor volumes, could be considerably reduced. However, a significant amount of pesticides could be sorbed on the wood in the RDB (Beltrán-Flores et al., 2021), which motivated the solid phase study presented in Section 3.4.

THIA was the pesticide with the lowest removal performances in both systems, being 38% and 80% for the FBR and RDB, respectively. In contrast, both the FBR (86%) and the RDB (100%) achieved high TEBU removals. Thus, although WRF have been reported to remove both pesticides (Chan-Cheng et al., 2020; Mori et al., 2021), in the present study *T. versicolor* was clearly more efficient removing TEBU than THIA.

Since the RDB showed the best results in terms of pesticide removal, as well as other parameters studied in Section 3.3.3, such as COD, colour and bacterial count studied in Section 3.3.3, a second batch was conducted in this system to evaluate its performance in a sequential treatment. In this regard, pesticide removal yields (87% total removal) comparable to those achieved in the first batch (85% total removal) were obtained, demonstrating the viability of the RDB for sequential/continuous treatments. The ability of the RDB to operate for extended periods has been previously reported for the treatment of spiked agricultural wastewater by (Beltrán-Flores et al., 2022), which was maintained for up to 225 days.

3.3.2. Toxicity

The capacities of both reactors to remove the pesticides detected in the AWW were evaluated in Section 3.3.1. However, other undetected compounds or potential metabolites may also contribute to the overall toxicity of the effluent (Marco-Urrea et al., 2009), thus acute toxicity and phytotoxicity analyses were also required (Table 1). Initial toxicity of 13.6 TU was considerably reduced in both reactors. As occurred in the case of the detected pesticides (see Section 3.3.1), sterile conditions favoured the reduction of toxicity in the FBR, denoting a better degradation activity of the fungal consortium in the absence of competing microorganisms. However, even under non-sterile conditions, the removal capacity of the RDB prevailed over that of the FBR-S. In any case, toxicity values were lower than the wastewater discharge limit (25 TU) established in Catalonia (DOGC, 2003).

Similar results were reflected in the phytotoxicity test. The RDB effluent caused less impact on tomato seed germination and growth than that of the FBR. Actually, the RDB effluent achieved even better SG and GI results than distillate water, which was probably ascribed to the release of some nutrients that considerably stimulate seedling growth,

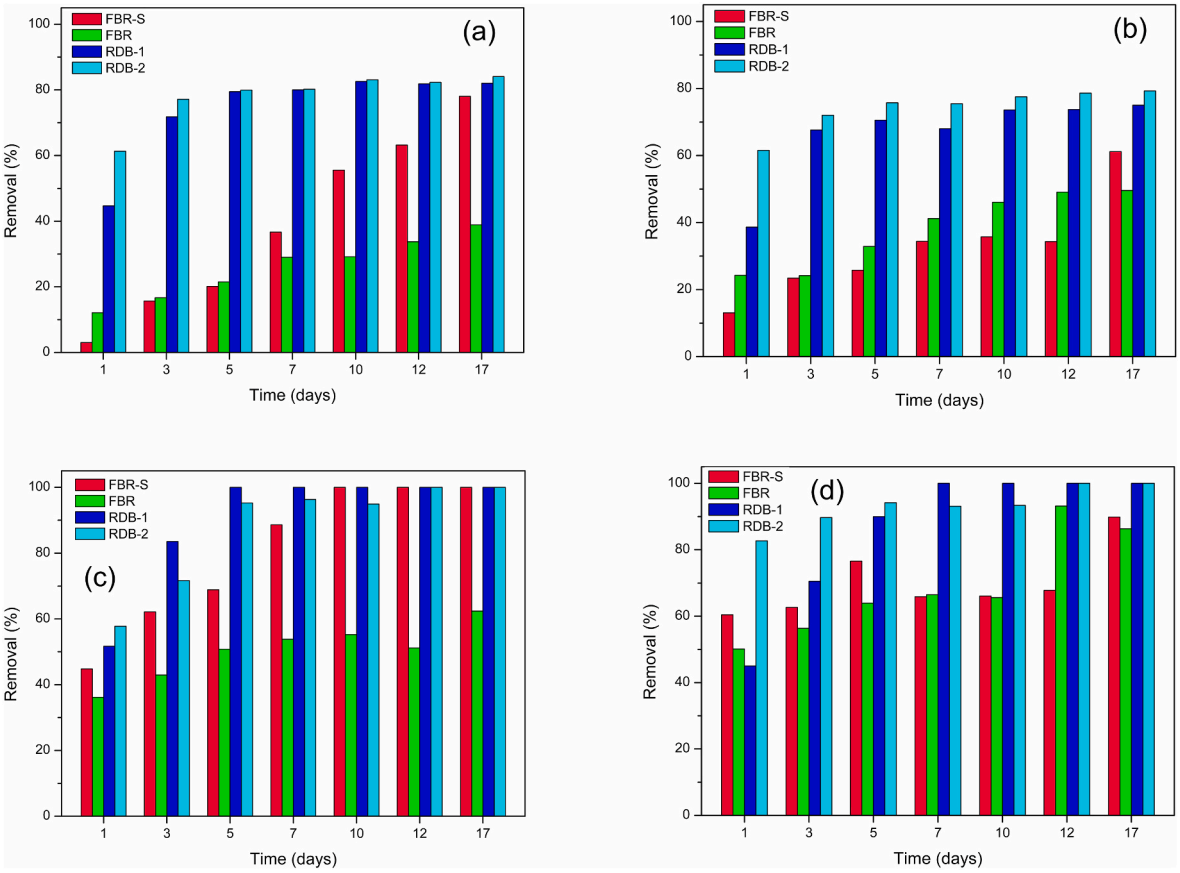

Fig. 1. Pesticide removals achieved for THIA (a), CHLOR (b), AZO (c) and TEBU (d) in each experiment.

Table 1
Toxicity and phytotoxicity of the treated and original agricultural wastewaters.

Wastewater	Operating conditions	Toxicity (TU)	Phytotoxicity		
			Relative seed germination SG (%)	Relative root elongation RE (%)	Germination index GI (%)
Pond	–	13.6 ± 1.0	52.9 ± 5.2	69.6 ± 4.7	36.9 ± 3.2
FBR-S	Sterile	6.0 ± 0.3	88.2 ± 20.6	82.28 ± 15.5	72.6 ± 17.8
FBR	Non-sterile	8.6 ± 2.8	76.5 ± 31.8	82.9 ± 11.9	63.4 ± 11.8
RDB-1	Non-sterile	2.2 ± 0.3	111.8 ± 40.9	93.7 ± 20.2	104.7 ± 29.2
RDB-2	Non-sterile	2.9 ± 0.1	61.8 ± 27.0	78.5 ± 1.7	48.5 ± 4.3

such as NH_4^+ and K^+ (Loffredo et al., 2016). However, partial inhibition was obtained in the RDB-2 compared to the RDB-1, indicating a certain deterioration of the effluent quality throughout the treatment. Since pesticide concentrations were even lower in the RDB-2 than in the RDB-1, the increased toxicity was related to the formation of some metabolites. In this respect, a possible metabolite of THIA with 200-fold higher toxicity in vertebrates has been previously reported (Casida, 2011). This phenomenon should be further investigated in future works.

3.3.3. *Monitoring of bioreactors*

As shown in Sections 3.3.1 and 3.3.2, the RDB was clearly more efficient than the FBR concerning toxicity abatement. However, there are other parameters to consider in selecting the best reactor for AWW treatment, such as laccase activity, COD, absorbance and microbial counts.

Laccase is a typical indicator used to evaluate enzyme activity in fungal treatments (Mir-Tutusaus et al., 2017). The RDB reached substantially higher laccase activity levels than the FBR during the treatment, with activity peaks of 16 and 2 AU·L^{-1}, respectively (Fig. 2). Furthermore, growing patterns were observed for the RDB and FBR-S,

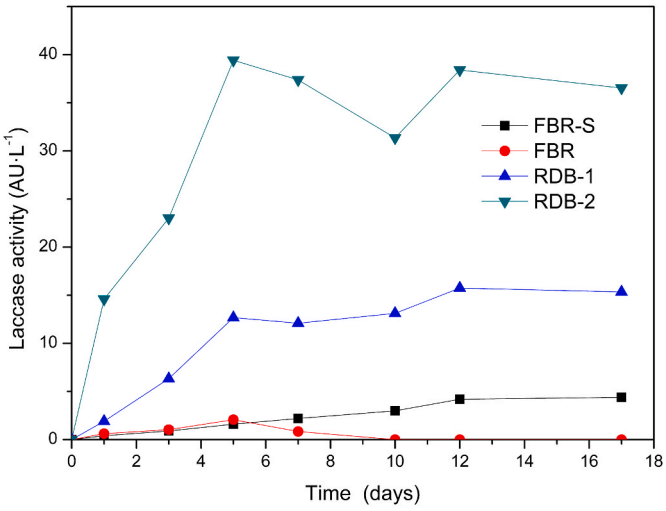

Fig. 2. Time-course profile of laccase activity in each experiment.

while the FBR showed a decreasing trend from day 5 onwards, evidencing the negative effect of contamination on the enzyme metabolism of the pelleted fungus. Interestingly, this laccase depletion was not observed in the RDB under non-sterile condition, indicating that the immobilization on wood chips played a vital role in maintaining the enzymatic activity of the fungal consortium.

Laccase production was mainly attributed to *Trametes* sp. and *Phanaerochaete* sp., which are the only two genera of WRF detected in the fungal consortium (see Section 3.5) capable of synthesising this enzyme (Yang et al., 2017). In this regard, Torán et al. (2017) reported low laccase activity for reactors based on both pelleted and colonized-wood, but in the latter case, lower extent of fungal colonization (according to ergosterol test) had been achieved during the fungal growth stage. Hu et al. (2021) obtained slightly higher laccase levels in an FBR with *T. versicolor* pellets compared to a trickle-bed reactor (TBR) with colonized wood, but this fact was mainly attributed to poor aeration inside the TBR. Furthermore, laccase activity was significantly higher in the second batch of the RDB, which evidenced that the immobilized fungal community was able to increase its enzyme activity as it adapted to non-sterile conditions. Laccase has been reported to be involved in pesticide degradation, although other enzymes or the intracellular P-450 enzyme system may also play a key role (Hu et al., 2022).

COD, absorbance and HPCs of the treated effluents are presented in Table 2. Contradictory results regarding the COD reduction capacity of *T. versicolor* have been previously reported (Cruz-Morató et al., 2013; Souza et al., 2014). In this study, COD increased significantly in both reactors, but to a lesser extent in the case of the RDB. In the RDB, the main contribution to COD was probably the release of organic matter from the wood chips (Lacorte et al., 2003). In the case of the FBR, COD increase was attributed to the addition of antifoam Tween 80 (also known as polysorbate 80, theoretical COD = 2207 mg O_2 mL^{-1}). In this respect, fungal treatments involving forced aeration are known to cause foaming (Font et al., 2003). Consequently, antifoam was only required in the FBR, as the RDB was an open system with aerobic conditions. More antifoam was required in the case of the FBR-S (7 mL) compared to the FBR (5 mL) probably owing to the more intensive metabolic activity demonstrated by *T. versicolor* under sterile conditions, which in turn increased COD level in the FBR-S. Regarding the RDB, COD was substantially lower in the second batch (RDB-2), which indicates that a significative part of the soluble organic compounds of the wood were extracted during the first cycle. Moreover, some of the soluble organic matter of the wood was probably consumed by *T. versicolor* and the associated fungal consortium instead of being released into the liquid phase (Beltrán-Flores et al., 2022). In that study, the COD of the inoculated reactor was found to be substantially lower than that of the control reactor, with this difference becoming more noticeable over time. Therefore, these results are a positive sign for the RBD application in large-scale during long-term operations.

Wastewater colouration increased throughout the treatment in the RDB, being substantially lower in the second batch (Table 2). As with the COD, the colour gain in the RDB was mainly attributed to the extraction of some organic compounds that give the characteristic dark colour to *Q. ilex* wood. In the case of the FBR, a higher turbidity was also observed probably as a consequence of a significant loss of pellet morphology (Espinosa-Ortiz et al., 2016).

The HPC results are presented as the logarithm of CFU in Table 2. The HPCs remained constant in both reactors, indicating that bacterial contamination was successfully controlled. The addition of glucose in the FBR did not result in significant bacterial proliferation, contrary to what was reported in the same reactor by Hu et al. (2021), probably caused by the inhibitory effect of the relatively high toxicity RW on the bacterial community.

Considering the remarkable good results of pesticide removal (Section 3.3.1), toxicity reduction (Section 3.3.2) and the stable values of the characteristic AWW parameters obtained in this section, the RDB effluent was found to present an overall quality clearly superior to that from the FBR and the original AWW. Furthermore, unlike the continuous glucose supplementation required in the FBR, the RDB only demanded the initial low-cost wood chips as substrate. Therefore, it is concluded that the RDB is the most promising treatment for further full-scale applications. This reactor could be used for the *in-situ* treatment of the AWW produced in the IRTA's agricultural fields (Mas Badía). In these fields, several pesticide applications are performed for each type of crop throughout the summer campaign. To avoid cross-contamination of pesticides between crops, the machinery is washed after each application, and the produced wastewater is accumulated in the collection pond. Accordingly, at the end of the campaign period, the collected wastewater could be treated in batch mode by a full-scale RDB for pesticide and toxicity abatement.

3.4. Solid-phase study and treatment

Studies involving sorption processes should not only evaluate micropollutant transfer from the liquid to the solid phase, but also the fate of the polluted sorbent. Hence, solid by-product obtained from the RDB were analysed to determine the contributions of the sorption and biodegradation to the overall removal by means of pesticide mass balance. Sorption was found to be a crucial mechanism of pesticide elimination, being around 81% THIA, 83% CHLOR, 83% AZO and 25% TEBU. This remarkable sorption contribution of pesticides by wood had previously been reported in the literature (Hu et al., 2021). Sorption capacity of wood has been related to pesticide hydrophobicity, and therefore, affinity of these compounds for wood fibres (Beltrán-Flores et al., 2021). In the present study, CHLOR and AZO showed slightly higher sorption than THIA, which may be due to the fact that they are more hydrophobic compounds. However, TEBU was the most apolar ($\log K_{ow} = 3.7$) and simultaneously the least sorbed pesticide, which was mainly attributed to its high biodegradability in the early stages of treatment, as shown in Fig. 1 (d). In other words, biodegradation was the fastest removal mechanism for TEBU, preventing its potential sorption on wood.

Solid by-products resulting from the RDB were subsequently treated

Table 2
COD, colour and microbial counts of the agricultural wastewater throughout the treatment in the three different fungal reactors.

Operation time (day)	COD (mg $O_2 \cdot L^{-1}$)					Colour					Microbial counts [log (CFU·mL^{-1})]			
	FBR-S	FBR	RDB_1	RDB_2		FBR-S	FBR	RDB_1	RDB_2		FBR-S	FBR	RDB_1	RDB_2
0	2088	2088	2088	2088	0.171	0.171	0.171	0.171	–		6.47	6.47	6.47	
0*	5190	4802							–					
1	5600	5160	3709	3228	0.079	0.183	0.197	0.156	–					
3	6380	7392	4407	3517	0.080	0.170	0.296	0.172	–					
5	8360	10,024	5340	3727	0.052	0.155	0.390	0.191	–		6.42	6.85	6.46	
7	11,448	10,404	6006	3754	0.046	0.129	0.413	0.197	–					
10	13,128	9296	6336	4068	0.039	0.212	0.427	0.228	–		6.48	7.02	6.10	
12	13,104	8120	6643	4158	0.040	0.310	0.302	0.247	–					
17	10,476	8140	7076	4785	0.041	0.372	0.243	0.212	–		6.61	6.54	6.51	

* Antifoam was initially added to prevent excessive foaming resulting from aeration.

in a biopile-like reactor during 27 days to evaluate the ability of the remaining fungus to biodegrade sorbed pesticides. Fig. 3 shows the results of the pesticide removal balance in the two-stage treatment consisting of the RDB and the biopile. The analysis of the biomass after the biopile treatment allowed the quantification of the pesticide biodegradation in the solid biopile, being 5% THIA, 26% CHLOR, 13% AZO, 2% TEBU. Although these percentages are apparently low, the total amount of pesticides removed is remarkable given their high content in the wood (≈28 mg g wood DW^{-1}). Thus, biodegradation rates of the pesticides were also calculated to facilitate comparison with other literature results (Table 3). In this regard, a similar biodegradation rate of total pesticides ($3.25{\cdot}10^{-4}$ mg g wood $DW^{-1}{\cdot}day^{-1}$) was achieved in a previous fungal treatment of wood in a biopile system (Beltrán-Flores et al., 2021). Longer operational periods, such as those commonly applied in compost-like systems, could considerably reduce the remaining pesticide content of the solid by-products (Rodriguez-Rodriguez et al., 2011). In addition, future research should explore other operational strategies to improve removal efficiency, such as the addition of fresh colonized wood, re-inoculation of solid by-products, adjustment of the moisture content, modification of the inoculation method and wood crushing.

3.4.1. Fungal community assemblage

In order to assess the fungal diversity of both reactors and the persistence of *T. versicolor* after being inoculated, a PCR-DGGE analysis was performed. The DGGE band profiles and UPGMA cluster analysis of the fungal populations from the effluent of the reactors can be observed in Fig. S1 (Supplementary materials). This figure shows remarkable differences between the final samples from both reactors, FBR and RDB, as they are clearly separated in the cluster analysis, while replicates of the same sample show a high degree of homogeneity. Besides, FBR samples cluster closer to the AWW, indicating fewer changes concerning the initial fungal community than after the RDB treatment.

To study the presence of *T. versicolor* in the effluent of both reactors, and rule out its previous presence in the AWW, prominent bands were excised from the gels and sequenced. This allowed determination of the phylogenetic affiliations of every recovered band and calculation of the relative abundance of each phylotype present in all samples analysed (Fig. 4 and Table S3, Supplementary materials).

Samples corresponding to AWW display high fungal diversity, with 3 prominent genera representing more than half of the fungal community (*Penicillium* sp., *Phanaerochaete* sp. and *Meyerozima* sp.). Both *Penicillium*

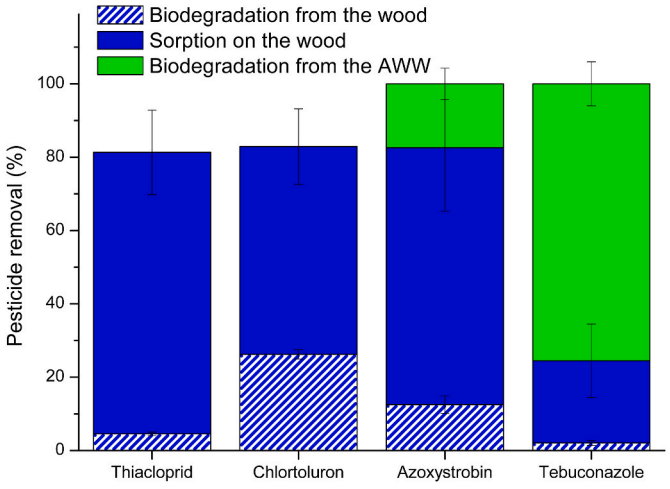

Fig. 3. Balance of pesticide removals in the RDB: biodegradation from liquid phase (green), adsorption on wood (blue) and biodegradation from wood in a biopile reactor (blue line pattern). Results are shown as mean values and corresponding standard deviations for triplicate measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Biodegradation of pesticides contained in polluted waste achieved by *T. versicolor* after 27 days of treatment.

Pesticide	Initial concentration ± SD ($mg{\cdot}g^{-1}$) x 10^2	Biodegradation ± SD (%)	Biodegradation rate ± SD ($mg{\cdot}g^{-1}{\cdot}day^{-1}$) x 10^5
Thiacloprid	3.83 ± 0.40	4.56 ± 0.47	7.96 ± 0.82
Chlortoluron	1.51 ± 0.12	26.20 ± 1.76	17.67 ± 1.19
Azoxystrobin	0.91 ± 0.14	12.54 ± 2.54	5.10 ± 1.03
Tebuconazole	0.57 ± 0.08	2.06 ± 0.91	1.78 ± 0.79

sp. and *Phanaerochaete* sp. have been reported to degrade pesticides (Chen et al., 2021; Zehra et al., 2017). *Meyerozyma* sp. has also been documented to degrade other micropollutants such as textile dyes (Ali et al., 2021). It should be noted that these samples present an array of minor bands that could not be studied due to their small representation (grouped under "Others"). Furthermore, *Trametes* sp. was not identified in any of the three replicates, indicating its absence from the initial AWW. Regarding those three prominent bands, *Penicillium* sp. is a ubiquitous genus, found as part of the mycobiome of many aquatic and terrestrial environments playing a paramount role as a decomposer (Park et al., 2018), and it has a major importance in the natural environment. The second one, *Phanaerochaete* sp., is of special interest since it is also a WRF with similar ligninolytic capabilities to *Trametes* (Köck-Schulmeyer et al., 2019). Finally, the yeast *Meyerozima* sp. has (among other capabilities) a Chitinase enzyme activity of great interest in plant growth promoting (Kapoor 2021).

Other genera detected in this initial sample include: *Rhodotorula*, one of the most studied yeast genera, that can utilize a wide range of substrates (mono- and polysaccharides, waste materials, etc.) while producing high-value substances (Kot 2016); *Didymella* (a fungal plant pathogen) (Corlett, 1981), *Cladosporium* (a common mold, occasionally found as a fruit pathogen) (Carolina Virginia et al. 2021), *Cystobasidium* (a parasite of other fungi, isolated from soils rich in cellulosic waste) (Vyas and Chhabra, 2017), and *Paraglomus* (a mycorrhizal fungus typically found in arable soils) (Hijri et al., 2006).

After treatment with RDB, the most obvious change in the community is the reduction of fungal diversity, with only 4 and 3 prominent bands left in the first and second batch samples respectively (Fig. 4). The appearance of *Trametes* is also noteworthy: it is detected in the effluent of the first batch, and its presence is increased after the second batch, representing in the end more than a 10% of the fungal diversity. However, when comparing with the fluidized reactor samples, it can be noted how the presence of *Trametes* sp. in the effluent of the RDB treatment is actually minor: in the final FBR effluent sample, *Trametes* sp. alone represents almost 50% of the fungal diversity. This result suggests that the immobilization on wood used in the RDB was more effective in retaining biomass than in the case of auto-immobilization (pellets) of the FBR. This statement is also supported by the clustering (dendrogram, Fig. S1) data that shows how the AWW community has a greater similarity to the FBR effluent community than to the RDB effluent samples. This indicates that fungal immobilization in the RDB allows for a greater change in the fungal community inside the reactor, influenced by the inoculated *T. versicolor*. However, in the FBR, although there is more presence of *Trametes* sp. in the liquid phase, the rest of the fungal community does not seem to have significantly changed, which may be due to a poor consolidation of *Trametes* sp. in pellet form, leading to a lower capacity for proliferation and influence on the rest of the species.

Finally, to better relate sample clustering with phylotypes present and environmental variables, a NMDS was plotted using DGGE band profiles and environmental parameters (colour, bacterial count ($CFU{\cdot}ml^{-1}$) and COD ($mg\ O_2{\cdot}L^{-1}$)) (Fig. S1 and Table S3). As can be seen from NMDS (Fig. 5), liquid samples show equivalent grouping to that observed in the dendrograms. Both RDB samples (first batch and second batch), appear close together in the plot, mostly influenced by three genera: *Rhodotorula*, *Meyerozyma* and *Cystobasidium*, and distantly

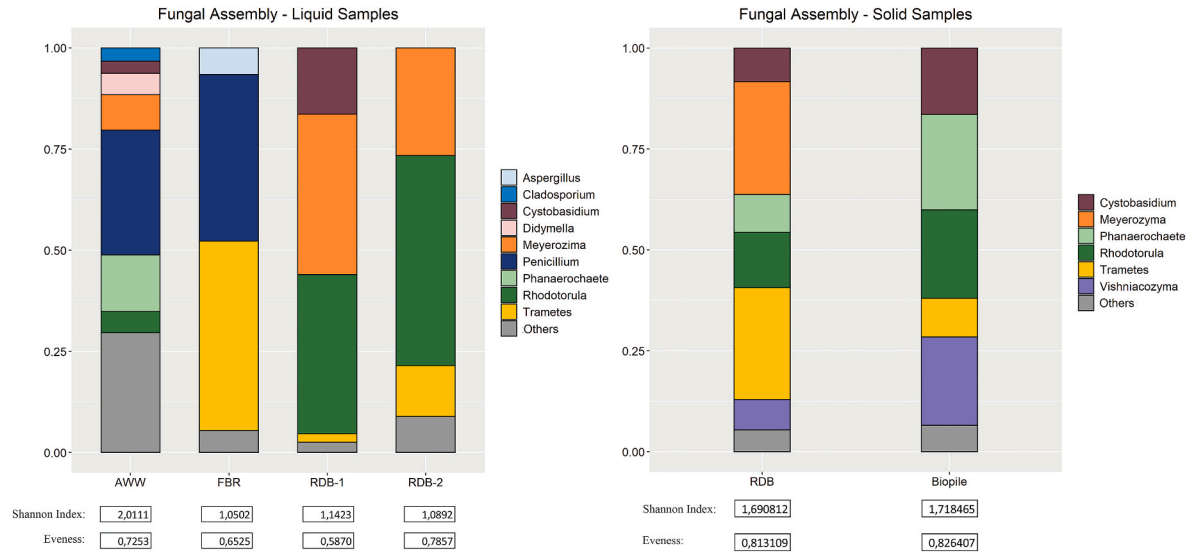

Fig. 4. Relative abundance of fungal populations in the original AWW and in the FBR and RDB effluents, corresponding to the liquid samples (left), and the RDB and the biopile lignocellulosic support samples at the end of each experiment (right). Values for Shannon Diversity and Evenness Indices are shown at the bottom of each sample.

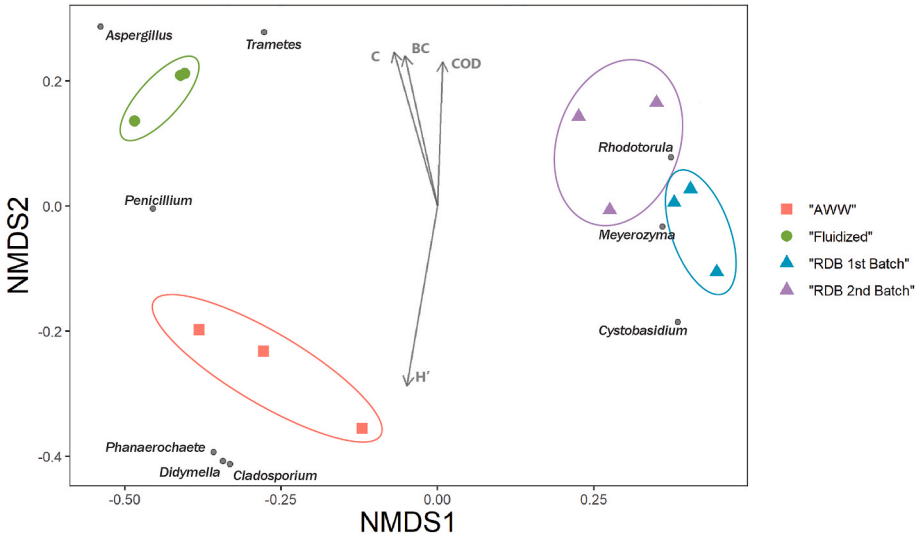

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination for all liquid samples based on Bray-Curtis similarity index with abiotic parameters and diversity indexes imaging. Symbols: red squares represent AWW, green circles correspond to final FBR effluent samples, and blue and lilac triangles belong to RDB effluent samples from the first and second batch, respectively. Grey dots represent species scores. Light grey vectors represent relevant (p.max = 0.05) parameters: C = "Colour", BC = "Bacterial Count", COD = "Chemical Oxygen Demand", H' = "Shannon Diversity Index). 2D Stress = 0.01764. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

related from AWW and FBR. In the same way, AWW and FBR samples form two separated groups depending on fungal populations composition and abundances, the former mostly influenced by differences in the community (involving *Phanaerochaete*, *Didymella* and *Cladosporium*), and the latter by *Aspergillus* and *Trametes*, respectively. All replicates of each sample seem to cluster together, indicating consistency in the results. Regarding environmental parameters, it can be observed how the AWW sample is greatly represented by the high values of their Shannon diversity indices. This fact can also be appreciated in Fig. 4, where it is shown how this sample has much greater fungal diversity (Shannon Index), as it was expected for an environmental sample, when compared to the "post-treatment" samples, both RDB and FBR effluents.

Wooden samples from RDB and the biopile were analysed together. DGGE band profiles and cluster analysis corresponding to the fungal community from extracted wood chips are shown in Fig. S1 (Supplementary materials). Samples from the RBD and the biopile appear completely separated and grouped with their corresponding replicas, as they are two different systems. When studying these solid samples, however, it must be noted that fungal diversity is relatively low, and similar fungal genera are detected in both systems (Fig. 4). Most bands seem to coincide in both treatments, with a significant difference: *Meyerozima* sp. seems not to be present in the biopile sample, although it was prominent in the RDB.

Interestingly, *Trametes* sp. decreases its presence in the community once the wood is treated in the biopile, while other genera seem to thrive and significatively increase their abundance. Those genera include *Phanaerochaete* sp. and *Cystobasidium* sp. (already mentioned in the previous section) but also *Vishniacozyma* sp., a cosmopolitan yeast, previously isolated from soil, wood, and fruit trees. Finally, values for Shannon Diversity and Evenness indices present no significant difference between them, indicating no major shifts in the community, aside from those already mentioned.

4. Conclusions

AWW with an inherently high pesticide content was treated in two different fungal reactors. The RDB proved to be a better candidate than the FBR according to all studied parameters, including 87% versus 51%

in pesticide removal, respectively. Fungal community study showed that *T. versicolor* was especially dispersed in the FBR, while this fungus was successfully immobilized in the RDB. In addition, solid by-products were treated with *T. versicolor* in a biopile reactor achieving remarkable biodegradation rates of pesticides of up to $17.67 \pm 1.19 \cdot 10^{-5}$ mg g^{-1} day $^{-1}$. These results suggest that the RDB is a promising approach for further AWW full-scale treatments.

Autorship contribution statement

Eduardo Beltrán Flores: Investigation, Formal analysis, Writing-original draft, Writing-Review & Editing. **Martí Pla-Ferriol**: Investigation, Formal analysis, Writing-original draft, Writing-review & editing of fungal community analysis. **Maira Martínez-Alonso**: Supervision, Conceptualization, Resources, Writing-review & editing of fungal community analysis. **Núria Gaju**: Supervision, Conceptualization, Resources, Writing-Review & Editing of fungal community analysis. **Montserrat Sarrà**: Supervision, Conceptualization, Resources, Writing-Review & Editing. **Paqui Blánquez**: Supervision, Conceptualization, Resources, Writing-Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.116595.

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